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Development of an analytical method for determination of urine 3-phenylpyruvic acid based on liquid-liquid extraction and LC-MS/MS

İdrarda 3-fenilpirüvik asit tayini için sıvı-sıvı ekstraksiyonuna ve Lc-Ms/Ms'e dayalı bir analitik yöntemin geliştirilmesi

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ABSTRACT

Aim: The aim of this study is to develop a rapid, precise, specific LC-MS/MS method for the determination of 3-phenylpyruvic acid, which has an important place in the diagnosis of phenylketonuria, a metabolic disease resulting from Phenylalanine Hydroxylase Enzyme deficiency.

Materials and Methods: Analytical measurements were made with Acquity UPLC MS MS (Waters Xevo TQD). The chromatographic separation was operated on an Acquity UPLC Phenyl column (50 mm × 2.1 mm, 1.7 µm) with gradient elution using 0.1% formic acid containing water and methanol as the mobile phase. Within the scope of the study, firstly, sample preparation steps were focused, and dispersive liquid-liquid extraction and traditional liquid extraction methods were examined. The best results were obtained in the conventional liquid-liquid extraction method, in which dichloromethane was used as the extraction solvent. Furthermore, trans-cinnamic acid molecule was used as an internal standard for the determination of 3-Phenylpyruvic acid in this study and tested according to the validation steps.

Results: The linear range of the developed LC MS MS method was found to be between 0.009-5 µM, while the detection limit was found to be 0.001 µM. Intraday repeatability was below 7% for 3 levels, and interlay repeatability was below 10%. In the recovery trial, which showed the accuracy of the method, the results in the urine sample for 3 levels were in the range of 97%-103%, and there was no significant ion suppression in the matrix effect trial, which showed how clean the final matrix obtained as a result of the sample preparation steps was.

Conclusion: As a result, a fast, inexpensive LC-MS/MS method has been developed for 3-Phenylpyruvic acid.

Keywords: 3-phenylpyruvic acid, trans cinnamic acid, LC-MS/MS, phenylketonuria, liquid-liquid extraction.

ÖZ

Amaç: *Bu çalışmanın amacı Fenilalanin Hidroksilaz Enzimi eksikliği sonucunda ortaya çıkan metabolik bir hastalık olan fenilketonürinin tanısında önemli bir yeri olan 3-fenilpirüvik asit tayini için hızlı, kesin, spesifik bir LC MS MS yöntemi geliştirilmesidir.*

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Gereç ve Yöntem: *Analitik ölçümler Acqutiy UPLC MS MS (Waters Xevo TQD) cihazı ile yapılmıştır. Kromatografik ayrım Acquity UPLC Phenyl (50 mm × 2.1 mm, 1.7 µm) kolonu ile metanol ve %0,1 formik asit içeren suyun mobil faz olarak kullanıldığı gradient elüsyon programı ile yapılmıştır. Proje kapsamında ilk olarak örnek hazırlama adımlarına odaklanılmış olup dispersiv sıvı-sıvı ekstraksiyon ve geleneksel sıvı ekstraksiyon yöntemi denenmiştir. En iyi sonuçlar diklorometanın ekstraksiyon çözgeni olarak kullanıldığı geleneksel sıvı-sıvı ekstraksiyon yönteminde elde edilmiştir. Ayrıca bu çalışmada 3-Fenilpirüvik asit tayini için internal standart olarak trans sinamik asit molekülü kullanılmış ve validasyon adımlarına göre test edilmiştir.*

Bulgular: *Geliştirilen LC MS MS yönteminin doğrusal aralığı 0.009-5* µ*M arası olarak bulunurken tayin limit ise 0.001* µ*M olarak bulunmuştur. Gün içi tekrarlanabilirlik 3 seviye için %7'nin altında, günler arası tekrarlanabilirlik ise %10'un altında bulunmuştur. Yöntemin doğruluğunu gösteren geri elde denemesinde ise gene 3 seviye için idrar örneğinde sonuçlar %97-%103 aralığında, örnek hazırlama aşamaları sonucu elde edilen son matriksin ne kadar temiz olduğunu gösteren matriks etkisi denemesinde kayda değer bir iyon baskılama olmadığı saptanmıştır.*

Sonuç: *Sonuç olarak 3-Fenilpirüvik asit için hızlı, ucuz bir* LC-MS/MS *yöntemi geliştirilmiştir.*

Anahtar Sözcükler: *3-fenilpirüvik asit, trans sinamik asit,* LC-MS/MS*, fenilketonüri, sıvı-sıvı ekstraksiyon.*

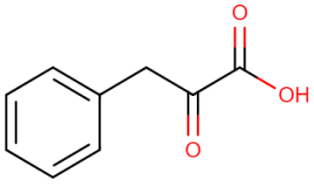
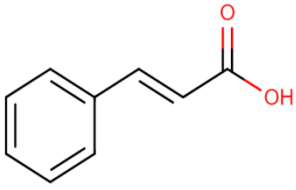
INTRODUCTION

Phenylpyruvic acid is a keto-acid that serves as an intermediate or catabolic byproduct of phenylalanine metabolism (1, 2). Normally, phenylpyruvate levels in blood or urine are very low. However, individuals with phenylketonuria (PKU), a congenital metabolic defect, may have high levels of phenylpyruvic acid in their urine (2, 3). PKU is caused by a deficiency of the enzyme phenylalanine hydroxylase (PAH), resulting in the conversion of phenylalanine to phenylpyruvic acid instead of tyrosine (2, 4, 5). People with PKU tend to excrete large amounts of phenylpyruvate, phenylacetate, and phenylactate, along with phenylalanine, in their urine (3). If left untreated, PKU can lead to mental retardation, microcephaly, unusual irritability, epileptic seizures, and skin lesions within the first year. Later in life, major clinical problems include hyperactivity, EEG abnormalities and seizures, and severe learning difficulties (4). Affected individuals also tend to have "musty or mouse" odors in their skin, hair, sweat, and urine (due to phenylacetate accumulation), as well as a tendency to hypopigmentation and eczema. The neural developmental effects of PKU are mainly due to the disruption of neurotransmitter synthesis. Specifically, phenylalanine is a large neutral amino acid that crosses the blood-brain barrier (BBB) via the large neutral amino acid transporter (LNAAT) (3). Excess phenylalanine in the blood saturates the carrier, thereby significantly reducing the levels of other LNAAs in the brain (6). As these amino acids are necessary for protein and neurotransmitter synthesis, the accumulation of phenylalanine impairs brain development, leading to mental retardation (7). Phenylpyruvic acid is also a microbial metabolite that can be produced by Lactobacillus plantarum. PKU is a disease that can be controlled with a diet low in phenylalanine, especially if diagnosed early. Therefore, early diagnosis of PKU and monitoring of its treatment are crucial.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS or alternatively HPLC-MS/MS) is an analytical chemistry technique that combines the mass analysis capability of mass spectrometry (MS) with the physical separation capabilities of liquid chromatography (or HPLC) (8). In short, it is an HPLC system whose detector is a tandem (sequential) mass spectrometer. What makes LC-MS/MS different from other HPLC techniques is its high precision and analytical specificity, making it useful for many applications. In the last 10-15 years, there has been a tremendous increase in the use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) in clinical laboratories. It has superior analytical specificity and higher yield than immunoassays and conventional high performance/pressure liquid chromatography (HPLC) for the determination of low molecular weight analytes. Most steroids and biogenic amines are now analyzed by LC-MS/MS all over the world, and the technology is also being used in smaller laboratories (9).

In LC-MS/MS methods, analytes cannot be accurately and precisely determined from the graph drawn with external standards due to effects such as losses in pretreatment and ion suppression (10). Therefore, it is necessary to use an internal standard that can tolerate losses by undergoing the same pretreatment as the target analyte (11). The internal standard used should have chemical and physical properties similar to the target analyte and should not be present in the sample being determined (9, 11). Molecules meeting these specifications are usually standards synthesized from stable isotopes of the target analyte. Stable isotopes have the same chemical and substantially similar physical properties as the target analyte but differ in molecular mass. The different molecular masses allow them to have a different multiple reaction monitoring (MRM) value in the MS/MS, thus enabling them to be determined independently of the target analyte. Although stable isotopes are used as internal standards in methods developed based on LC-MS/MS, molecules that are structurally very similar to the analyte, such as analogues of drugs, can also be used. Trans-cinnamic acid is a phenolic compound found in foods and is not synthesized endogenously in humans. Trans-cinnamic acid is not found within the limits of quantitation in urine (12). One of the questions that this study seeks to answer is whether it can be used as an internal standard for the determination of trans-cinnamic acid. Table-1 shows the physical and chemical properties of 3-phenylpyruvic acid and trans-cinnamic acid. As can be seen from the table, the solubility and acidity-basicity constants of 3-phenylpyruvic acid and trans-cinnamic acid, which are both phenolic compounds, are very close to each other (13, 14).

Table-1. Physicochemical properties of 3-phenylpyruvic acid and trans cinnamic acid.

	3-phenylpyruvic acid	**Trans cinnamic acid**
Systematic Name	3-Phenyl-2-oxopropanoic acid	3-Phenyl-2-propenoic acid
Formula		
Molecular weight (g/mol)	164.158	148.1586
Solubility in water	0.93 mg/mL	0.62 mg/mL
LogP	1.9	2.13
pKa	3.33	4.51
H acceptor	3	2
H donor	1	1

When examining the methods developed for 3-phenylpyruvic acid in the literature, a few methods were found: the colorimetric method (15), fluorometric method (16), the HPLC method (17–19), LC-MS/MS method (20) and a cell-based biosensor method (21). Among these methods, the HPLC method includes a derivatization step that prolongs the sample preparation process, while the colorimetric method is susceptible to interference. The biosensor method is not suitable for multiple analyses and has reproducibility issues.

The aim of this study is to develop a derivatization-free LC-MS/MS method for 3-Phenylpyruvic acid. One of the most important factors that increase the cost of LC-MS/MS methods is the presence of isotope-labeled internal standards required for quantitation. In this study, we aimed to find a solution to this problem by using trans cinnamic acid, which is a non-isotope-labeled, exogenous, inexpensive, and physiochemically similar molecule to 3-phenylpyruvic acid. This study is the first to be developed for 3-phenylpyruvic acid and used

trans cinnamic acid as an internal standard, which is one of the factors that contribute to the originality of the project. Another unique feature of the project is the sample preparation phase. Both the dispersive liquid-liquid extraction method and the traditional liquid-liquid extraction method were tried, and the best extraction method was selected.

MATERIALS and METHODS

Chemicals

3-Phenylpyruvic acid and trans cinnamic acid were supplied from Sigma (St. Louis, MO, USA). Acetonitrile, methanol, acetone, dichloromethane, ethyl acetate, diethyl ether, chloroform, HCl and formic acid, each of them LC-MS grade solvents, were provided by Merck-Lichrosolv (Darmstadt, Germany). LC-MS grade water was purchased from AppliChem (Darmstadt, Germany). Liquichek Urine Chemistry Control was purchased from Biorad. The Liquichek Urine Chemistry Control is a liquid control product made from human urine and is designed to assess the accuracy of urine chemistry testing methods. It has been assayed for this purpose. This commercially available urine product is obtained by lyophilizing human urine. Its contents include the following analytes that are present in healthy human urine: calcium, chloride, creatinine, glucose, magnesium, microalbumin, phosphorus, potassium, sodium, urea, and uric acid. Stock solutions of 3-phenylpyruvic acid and trans cinnamic acid standards were prepared in methanol at a concentration of 1 mM. Dilutions were made with Sigmatrix Urine Diluent (Sigma Aldrich, USA) urine diluent. Sigmatrix urine diluent containing calcium chloride, magnesium chloride, potassium chloride, sodium chloride, sodium phosphate, sodium sulfate, urea, and creatinine with sodium azide as a preservative.

Sample Preparation

500 µl of 0.1 M HCl is added to 500 µl of commercially purchased urine, followed by the addition of 200 µl of internal standard (5 µg/ml trans cinnamic acid) and brief vortexing. Subsequently, 1000 µl of extraction solvent dichloromethane is added and vortexed for 30 seconds. The mixture is then centrifuged at 10000 g for 10 minutes, and the organic solvent phase is transferred to a plate and evaporated under nitrogen gas. Finally, 100 µl of methanol is added to the evaporated samples, and they are re-dissolved before being subjected to analysis by LC-MS/MS.

UPLC Conditions

Optimization of UPLC conditions consists of column selection, mobile phase composition, flow rate, injection volume optimization. Analysis was performed on a A Waters ACQUITY Xevo TQD system, consisting of an ACQUITY UPLC system and an ACQUITY triple-quadrupole tandem mass spectrometer with an electrospray ionization (ESI) interface (Waters Corp., Milford, MA, USA). Separation of analytes was achieved using a Acquity BEH Phenyl Column (50 × 2.1 mm, i.d., particle size 5 µm; Chiral Technologies, Illkirch, France) with a Chiralpak® IF (50 mm × 2.1 mm, 1.7 µm). The mobile phases consisted of Methanol (A) and water containing 0.1% formic acid (B). Chromatografic separation was performed in gradient elution mode at a flow rate of 0.40 mL/min with an injection volume of 5 µL. The gradient started at 5% A, held for 1.00 min, then increased to 100% A until 2.0 min and this was maintained for 1.00 min before the gradient finally returned to 5% A at 3.00 min until 4 min to re-equilibrate the column.

MS MS Conditions

The negative ion mode of electrospray ionization (ESI) was chosen for analyses. Quantification was achieved using the multiple reactions monitoring (MRM) mode. The optimal conditions for the ESI source were as follows: the capillary voltage was set at 2.82 kV, the source temperature was 150 °C, the desolvation gas temperature was 350 °C, the cone gas flow rate was 40 L/h, and the desolvation gas flow rate was 600 L/h. The MRM transitions were 162.81/90.87 for 3-FP, 147.00/ 103.10 for Trans cinnamic acid (IS), respectively. Data acquisition was performed using MassLynx 4.1 software with the Targetlynx program (Waters Corp., Milford, MA, USA).

Sample Preparation Pre-Treatment Optimizations

In order to examine the effect of sample preparation on the determination of 3-phenylpyruvic acid in urine, dispersive liquid-liquid extraction method and traditional liquid-liquid extraction methods were tried. For the dispersive liquid liquid extraction method, 200 µl of dichloromethane, chloroform, ethyl acetate and diethyl ether were tested as extractant solvents in 1 ml of urine sample, and 1 ml of acetonitrile, methanol and acetone were tested as dispersion solvents. In the traditional liquid liquid extraction method, 1 ml dichloromethane, chloroform, ethyl

acetate and diethyl ether were examined as extraction solvents for 500 µl urine sample.

Method Validation

Method validation studies were carried out in accordance with the bioanalytical method development and validation guide of the European Medicines Agency (EMA), and in this context, the selectivity, linearity, sensitivity, recovery and matrix effect of the method were determined (22).

Selectivity

Internal standard and 3-Phenylpyruvic acid added standard matrix and urine matrix were studied 6 times. The answers obtained were evaluated by comparing them according to the method validation criteria (22).

Determination of Linear Range and Calibration Graph

The linear detection range and calibration curve of the developed method were determined. In this study, the experiment was carried out with a trans cinnamic acid concentration of 5 µg/ml and 3-phenylpyruvic acid (3-FP) concentrations of 10 points between 0.009-5 µM to determine the linear determination range. The assay was performed by adding the standard to the urine. The results obtained were plotted as 3-FP concentrations versus 3-FP/internal standard peak area ratios. Each measurement was repeated three times.

Linearity and sensitivity

The limit of determination and quantitation was calculated using the slope and standard error of the calibration plot. The relevant equation is: LOD = 3xStandard error of graph/slope; LOQ=3.3xLOD (23, 24).

Precision

The reproducibility study was performed for 3-FP by running 10 intra-day and inter-day measurements at concentrations of 0.1, 1, and 5 µM.

Recovery and matrix effect

Accuracy refers to the proximity of the obtained values to the actual values. Recovery studies were conducted for urine samples at three different concentrations, with at least five replications for each concentration (18). Points representing the lowest, middle, and highest concentrations of the linear range were carefully selected. After the recovery studies were conducted at 3-FP concentrations of 0.1, 1, and 5 µM, the average percent recovery rates were calculated.

RESULTS

Phenylpyruvic acid and Transcinnamic acid chromatograms

Method development studies for 3-Phenylpyruvic acid in urine began with optimizing the MS conditions and determining MRM values. We worked in both negative and positive ion modes, but the best signals were obtained in negative ion mode. The results of the MS parameter optimization are given in Section 2.4. We optimized the MS parameters using 5 µg/ml solutions of 3-phenylpyruvic acid and trans cinnamic acid prepared in methanol.

After optimizing the MS and MRM conditions, the second step was determining the UPLC conditions. To do this, we tested solvents of water, methanol, and acetonitrile in the mobile phase. Our aim was to obtain the most symmetrical and highest peak response, so we tried the isocratic method with varying ratios of the water phase from 100% to 0% and the methanol phase from 0% to 100%. We observed that the peak was very late and broad in the water phase, whereas it was sharp, symmetrical, and high in the methanol phase. Similar results to those obtained with methanol were also obtained with acetonitrile. Consequently, we determined that both 3-phenylpyruvic acid and trans cinnamic acid, the internal standard, were better in the organic solvent phase. Since methanol is cheaper than acetonitrile, we chose methanol as the organic solvent for the mobile phase. We initially conducted experiments using the UPLC BEH C18 column. Later, we tried the BEH Phenyl column and found that the peak intensities and symmetry obtained with the Phenyl column were better. When examining the structure of both 3-phenylpyruvic acid and trans cinnamic acid, we noticed that they both have a phenyl ring. This suggests that both molecules are better retained in the column and can be easily separated from other ions to be suppressed. To minimize interference and ion suppression effects, we kept the molecules in the column for a while, resulting in healthier results. For this reason, we chose the gradient method as the UPLC flow program, with the gradient starting in the 100% water phase for 1 minute, then transitioning to 100% methanol for 1 to 2 minutes and continuing with the 100% methanol phase for 2 to 3 minutes. In this way, substances that could create a matrix effect were flushed out of the column first, preventing any interference effect they might create. By using

formic acid in the water phase, we achieved pH stabilization and protonated the carboxylic acid groups of the molecules. While the polarity of protonated molecules decreases, their solubility in water also decreases, leading to increased solubility in organic solvents and resulting in a clean peak.

The representative chromatograms of 1 µM 3-Phenylpyruvic acid and 5 µg/ml Trans cinnamic acid incorporated into the urine matrix are shown in Figure-1. If you pay attention to the figure, both molecules elute from the column at close retention times due to their similar chemical structures. The retention time of trans cinnamic acid was found to be 2.28 minutes, while the retention time of 3-Phenylpyruvic acid was 2.05 minutes.

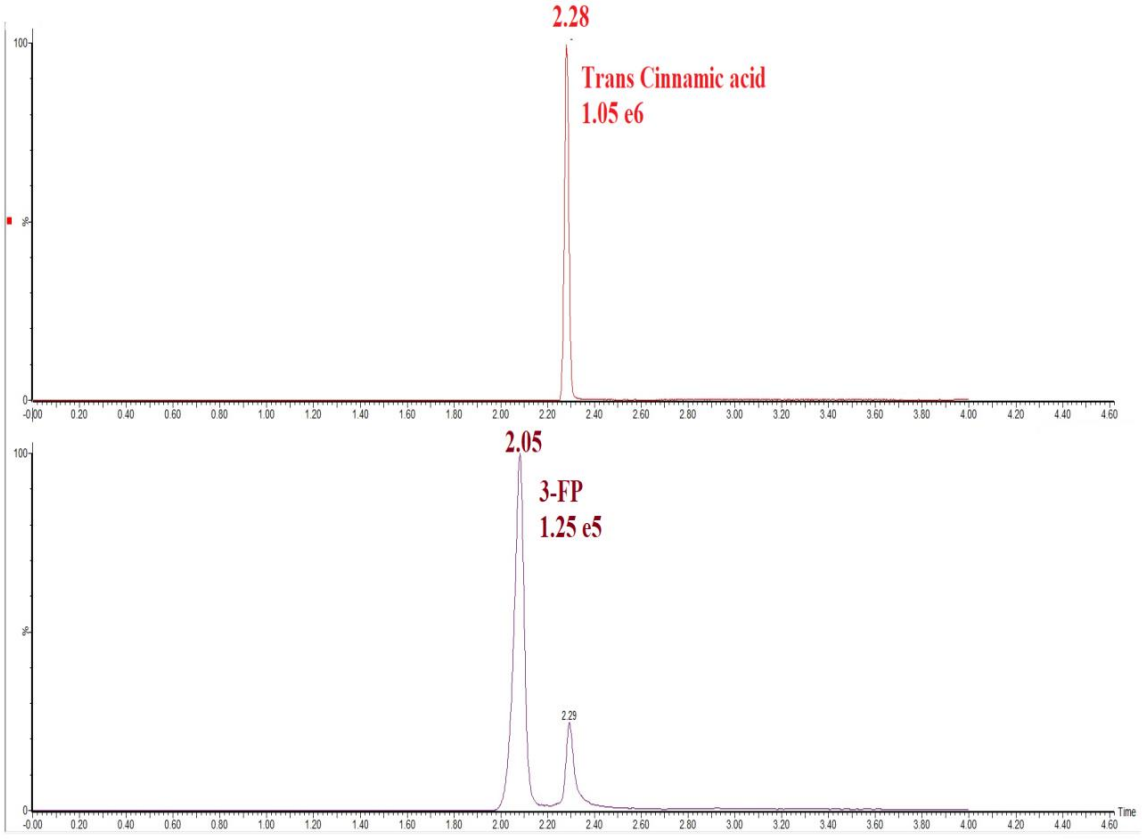


Figure-1. Representative chromatograms of 3-phenylpyruvic acid and trans cinnamic acid in urine matrix.

Determination of extraction method

Extraction methods are crucial in sample preparation for LC-MS-MS based methods, in order to reduce ion suppression effects and provide a cleaner matrix to the instrument. For this purpose, both a dispersive liquid-liquid extraction method that requires less solvent and a traditional liquid-liquid extraction method were tested for 3-phenylpyruvic acid. Figure-2 shows the results of the dispersive liquid-liquid extraction method, where the best results were obtained using a solvent mixture with dichloromethane as the extraction solvent and acetone as the dispersing solvent. In the system using a dichloromethane-methanol mixture, phase separation was not completely achieved. In the traditional liquid-liquid extraction method, the best result was obtained using dichloromethane as the extraction solvent. Additionally, the peak intensity obtained by the traditional method was approximately three times higher than that obtained by the dispersive method ($p=0.0075$, t test). Therefore, the traditional liquid-liquid extraction method using dichloromethane as the extraction solvent was selected as the optimum extraction method.

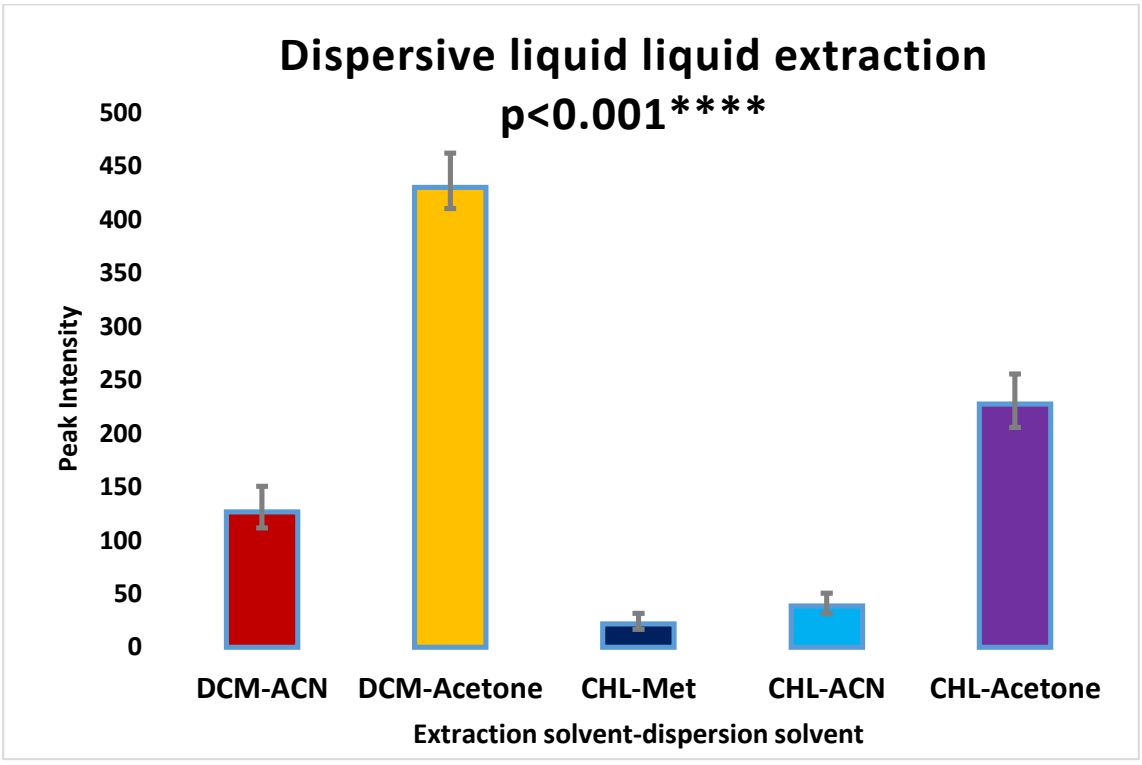


Figure-2. Dispersive Liquid Liquid Extraction Experiment Results for 3-Phenylpyruvic acid determination. ANOVA test was used in the comparison between methods (significance level p<0.05).

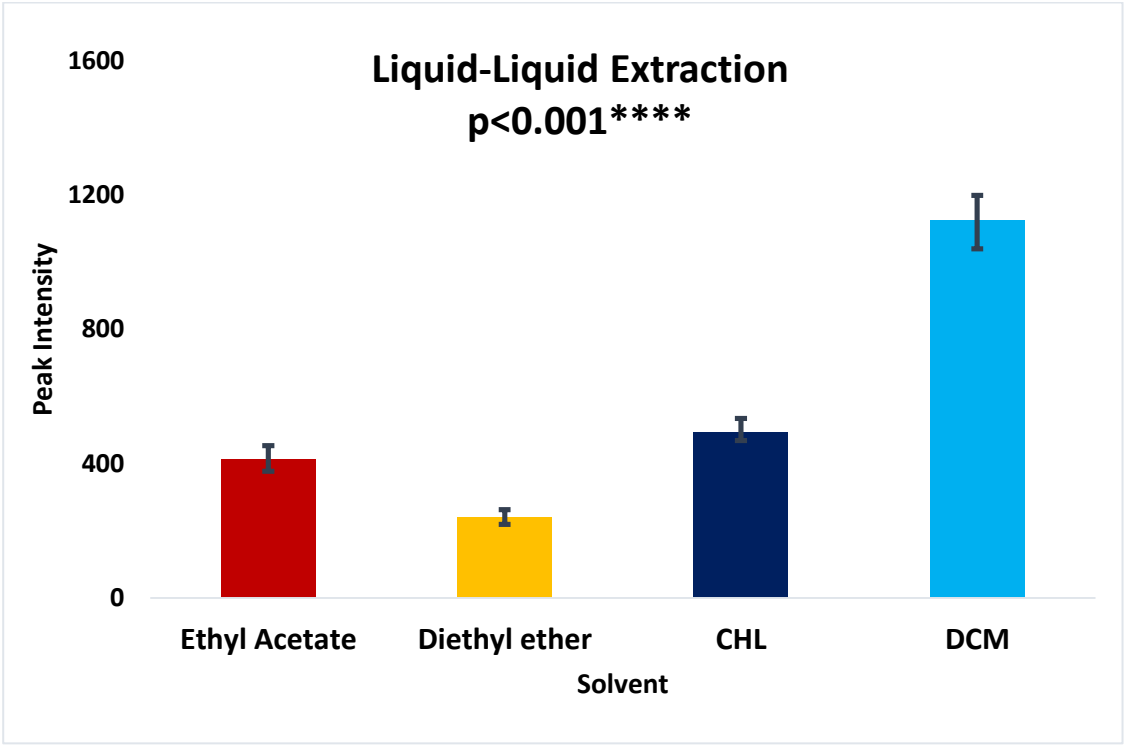


Figure-3. Traditional liquid liquid extraction experiment results for 3-Phenylpyruvic acid determination. ANOVA test was used in the comparison between methods (significance level p<0.05).

Selectivity

In order to determine the selectivity of the method, peak areas of the MRM chromatograms of 3-phenylpyruvic acid and trans-cinnamic acid were examined in both blank urine matrix and mobile phase. The selectivity of the internal standard in urine matrix was tested 6 times and the % interference values in Table-4 were obtained by dividing the peak areas obtained without adding the internal standard by the peak areas obtained after adding the internal standard and multiplying by 100.

According to the validation criteria, the interference effect detected for the internal standard should be below 5% of the response of the internal standard used, and the interference effect detected for the analyte should be below 20% of the response at the quantification limit. As shown in Table-4, the % interference obtained for Trans Cinnamic Acid was found to be 0.108%, while the interference for 3-Phenylpyruvic Acid was found to be 1.03%. These values are within the validation criteria.

Linear Range

The linear range of the developed method was determined in urine matrix. Urine matrix containing 0.009, 0.019, 0.038, 0.076, 0.152, 0.325, 0.625, 1.25, 2.5, and 5 µM of 3-Phenylpyruvic acid was prepared according to the sample preparation procedure described in Section 2.2. Figure-4 shows the urine matrix chromatograms, while Figure-5 shows the calibration curve, calibration equation, and R^2 values. According to validation criteria, the linear determination range should consist of at least 6 points, and the R2 value of the graph should be greater than 0.985. As shown in Figure-5, the graph consists of 10 points, and the R^2 value is 0.9908. The method's quantitation limit is 0.009 µM, and the detection limit is 0.001 µM.

Table-2. Selectivity results of trans cinnamic acid (IS) and 3-Phenylpyruvic acid in the urine matrix.

	Peak intensity of spiked analytes in urine matrix	**Blank urine matrix**	**% Interference**
Trans cinnamic Acid (5 ug/mL)	4238.21 ±115.1	4.57±0.251	0.108±0.001
3-FP (10 nM)	987.82 ±83.99	10.19±3.51	1.03±0.06

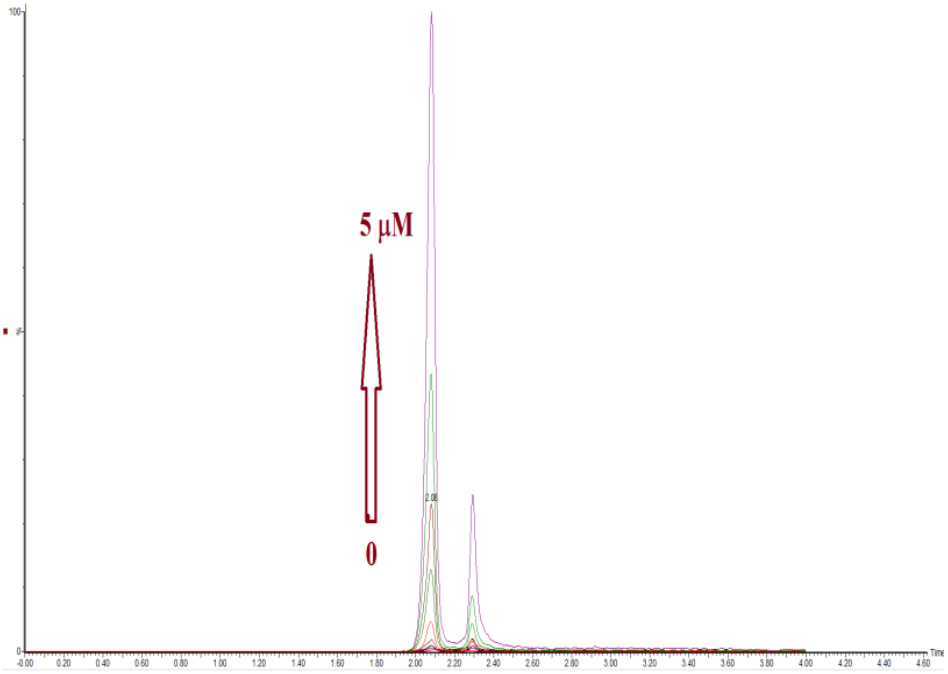


Figure-4. Chromatograms for 3-Phenylpyruvic acid between 0-5 µM in the urine matrix.

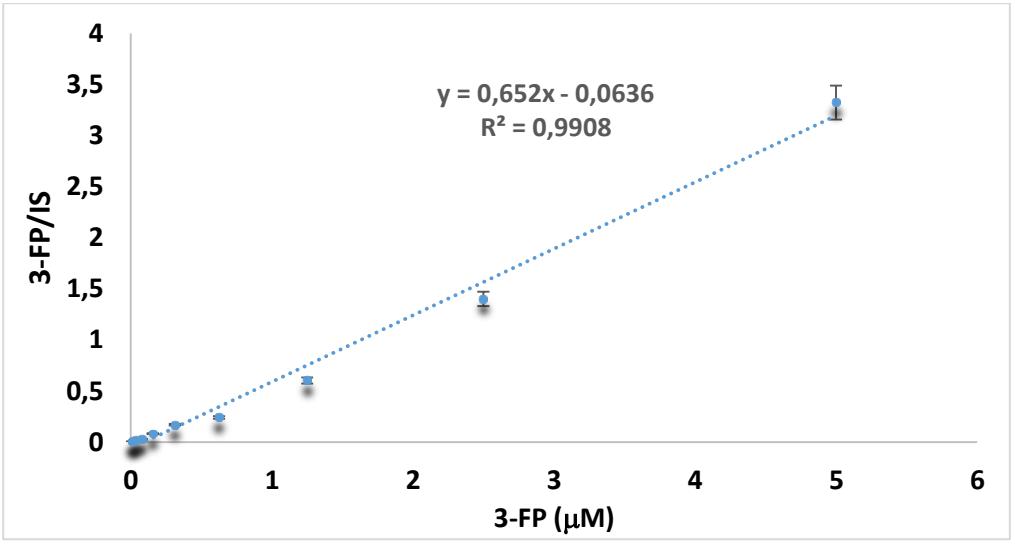


Figure-5. Standard plot of 3-Phenylpyruvic acid between 0-5 µM in the urine matrix.

Table-3. Evaluation of the within run and between run precision for the determination of 3-FP.

	Interassay (n=10)		Intrassay (n=10)	
	Mean ± SD	**CV%**	**Mean ± SD**	**CV%**
0.1	0.102±0.0086	6.50	0.109±0.012	10.46
1	0.982±0.063	6.14	1.07±0.104	9.64
5	5.117±0.316	3.12	5.78±0.32	3.32

Table-4. Results of recovery and matrix effect of the method.

3-FP (□M)	**Recovery % (n=5)**	**Matrix Effect % (n=5)**
0.1	% 103.75	% 90.41
1	% 98.41	% 95.41
5	% 99.71	%97.3

Table-5. Comparison of reported 3-FP determination methods.

Methods	**Matrix**	**Detection limit**	**Extraction solvent**	**Derivazation**	**Ref.**
Colorimetric	Urine	0.3 mM	Diethyl ether	Yes	(15)
Fluorometric	Urine	2.0 µM	Diethyl ether	Yes	(16)
HPLC with fluorescence detector	Serum	160 nM	Ethyl acetate	Yes	(17)
	Urine	125 nM	Ethyl acetate	Yes	
HPLC with chemiluminescence detector	Plasma	9 nM	Anion exchange cartridge	Yes	(18)
HPLC with fluorescence detector	Urine	40 nM	6 M HCl	Yes	(19)
LC MS MS	Plasma	50 nM	Acetonitrile/NaOH (0.1 %, wt) (v/v, 1/1)	Yes	(20)
LC MS MS	Urine	1 nM	Dichloromethane	No	This work

Precision

The precision of the method, which refers to its repeatability, was tested intra-day and inter-day. Table-3 shows the mean, standard deviation, and % CV values for three concentrations: low, medium, and high. Since values below 15% are considered acceptable, the obtained values are within an acceptable range.

Recovery and matrix effect

The recovery and matrix effect studies were performed at three points representing low, medium, and high concentrations within the linear range, with five repetitions. The % Recovery Results, standard deviation, and % matrix effect values of the developed method are shown in Table-4. The results of the recovery experiment, which indicates the accuracy of the method, are quite satisfactory. The matrix effect experiment, which shows how clean a matrix is obtained for the analyte as a result of the pre-treatment, revealed that the ion suppression effect is approximately 10% for low concentrations and around 3% for high concentrations.

Comparison of reported 3-FP determination methods

Comparison of the three existing 3-FP determination methods in the literature is shown in Table-5. When the developed methods are examined, it can be seen that the sensitivity of colorimetric and fluorescence-based methods increased when combined with HPLC. Diethyl ether, ethyl acetate, HCl, or acetonitrile/NaOH were used as extraction solvents. Derivatization method was used to increase sensitivity in the existing methods in the literature. The derivatization step increased the sensitivity but extended the method time with an additional sample preparation step. 3-Phenylbutyric acid was used as an internal standard in HPLC methods, while isotope-labeled standards were used in LC MS MS method. This study has better sensitivity and shorter sample preparation time compared to other methods. At the same time, ethyl acetate and diethyl ether, which were used as extraction solvents in the existing studies in this work, were also tested and it was found that dichloromethane had a better extraction yield from both solvents (See Figure-3).

DISCUSSION

In this study, a new LC MS MS-based method has been developed for the determination of urine 3-phenylpyruvic acid levels, which are necessary for the diagnosis and monitoring of phenylketonuria. Firstly, the best extraction method was investigated using the developed method. Then, the suitability of the developed method was tested according to the method validation criteria. One of the questions this project sought to answer was whether trans-cinnamic acid could be used as an internal standard. As a result of LC conditions optimization, the mobile phase was determined to be composed of methanol as phase A and 0.1% formic acid in water as phase B, with a flow rate of 0.4 mL/minute and a gradient system flow program. The total measurement time was 4 minutes, with retention times of 2.05 and 2.28 minutes for 3-phenylpyruvic acid and internal standard trans-cinnamic acid, respectively. The relatively larger size of the nonpolar portion of 3-phenylpyruvic acid and trans-cinnamic acid compared to the polar portion explains why they come in the methanol phase. The best results were obtained using the traditional liquid-liquid extraction method with dichloromethane in conjunction with this study. While organic solvents used for extraction in the literature were diethyl ether (15, 16) and ethyl acetate (17) this study revealed that dichloromethane further improved the results. The extractant volume used in the traditional liquid-liquid extraction method is 10 times greater than that used in the dispersive liquid-liquid extraction method. Therefore, higher peak responses may have been obtained with the traditional liquid-liquid extraction method. While the DLLME method has been successfully applied to more hydrophobic and larger molecules such as pesticides or steroids it has not been widely used for smaller and amphipathic molecules (25, 26). In this study, both methods were compared, and it was found that the traditional liquid-liquid extraction method was better for determining 3-FP in urine. Trans-cinnamic acid is a phenolic compound that is very similar to 3-phenylpyruvic acid in physical and chemical properties. It is not synthesized endogenously but can be obtained from food and is not typically found in urine. These findings support our thesis that trans-cinnamic acid can be used as an internal standard. Additionally, no peak was found for trans-cinnamic acid in lyophilized urine matrices from commercially available kits used in our study. The peak response of trans-cinnamic acid in the samples is

equivalent to an average of 0.1% of the peak response of 5 µg/ml trans-cinnamic acid. Acceptable values are 5% or lower; therefore, trans-cinnamic acid can be used as the internal standard for 3-phenylpyruvic acid. In this study, the linear range of the method we developed for urine matrix is 0.009-5 µM and the detection limit is 0.001 µM. The % recovery experiment was performed for low, medium, and high concentrations of 3-Phenylpyruvic acid, which represent 0.1-1-5 µM, respectively. The % recovery values for these concentrations were 103.75%, 98.41%, and 99.71%, respectively. The % matrix effect values for 0.1-1-5 µM 3-Phenylpyruvic acid concentrations in the method we developed were 90.41%, 95.41%, and 97.3%, respectively. The obtained values indicate that the developed method produces highly accurate results and also shows that a clean matrix is obtained as a result of the extraction stage.

CONCLUSION

In this project study, the method we developed has a shorter pre-processing time, a faster analysis time, and lower detection and quantification limits compared to other methods and is the first 3-Phenylpyruvic acid LC-MS/MS determination method in the literature using the dichloromethane liquid-liquid extraction method and trans-cinnamic acid as an internal standard.

Conflicts of interest: Authors declared no conflict of interest.

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